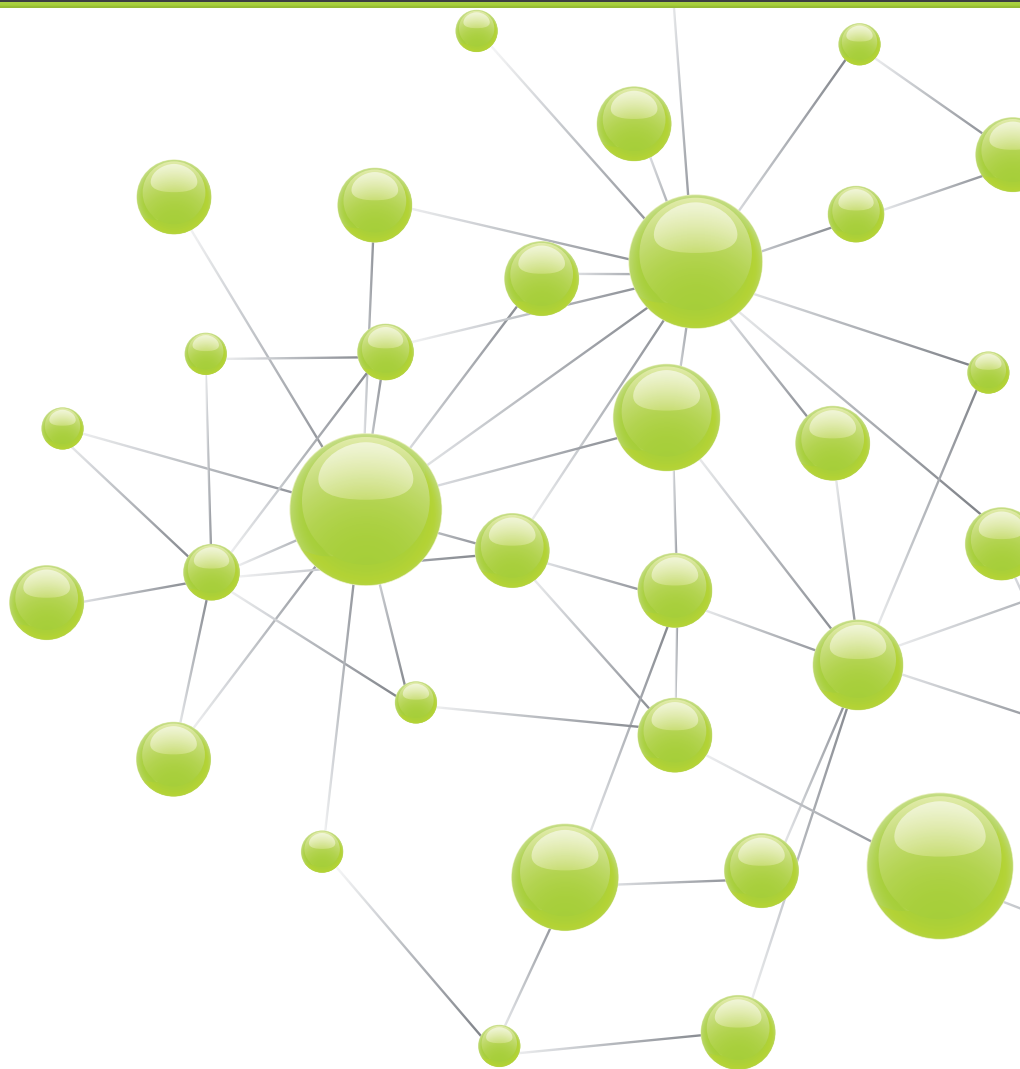




nCounter®

Gene Expression Assay User Manual

Total RNA and Cell Lysate Protocols

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Molecules That Count®

Translational Research • Gene Expression • miRNA Expression • Copy Number Variation

MAN-C0003-03

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PREFACE

Conventions Used

The following conventions are used throughout this manual and are described below for your reference:

Note Types

Special font formatting is used in this manual. Such formatting conventions are used in specific instances as described below:

TIP		Information contained in a Tip may offer helpful suggestions, alternative procedures, methods and/or shortcuts.
NOTE		This note type emphasizes general information.
IMPORTANT		This note type presents essential content indicating that the potential exists for assay failure, diminished data quality, and/or a loss of data if the information presented is ignored.
WARNING		This note type indicates that a potential hazard to your personal safety, or the potential for equipment damage exists.
BOLD		When appearing in text or in a procedure, the bold text serves to highlight a specific button, key stroke, or menu option available. • Bold text may appear elsewhere to highlight important text or terms. • Green text is used to help the reader identify active hyperlinks.
ITALICS		Used to emphasize an important word or expression within the text. • Formatting of a book title, journal, or other documentation. • Used to indicate the special or unusual meaning of a word or phrase.

Procedures

Numbered procedures appear frequently providing step-by-step instruction for accomplishing a task. Typically, a numbered step provides direction for a specific action and may be followed by the expected response. Additional information may be presented in the form of a specific note type, bullets, screen capture or other image important to facilitate clarity and understanding. For example:

In the (next) screen, the active data entry field is indicated by a green box around it. Simply move from one field to the next, simply press the desired field on the touchscreen with your finger.

1. To add an email address, press **ADD**.
>>> The email address keyboard screen appears.
2. Enter a valid email address and press **ENTER**. The email address gets saved.

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1 Introduction

Introduction

The **nCounter® Gene Expression Assay** is designed to provide a sensitive, reproducible and highly multiplexed method for detecting gene expression across all levels of biological expression. This assay provides a method for detecting mRNAs with molecular barcodes called nCounter Reporter Probes without the use of reverse transcription or amplification.

This manual describes in detail the methods for setting up nCounter hybridization assays. Please see the **nCounter® Prep Station User Manual**, **nCounter® Digital Analyzer User Manual**, and **nCounter® Data Analysis Guidelines** for instructions on post-hybridization processing and data analysis.

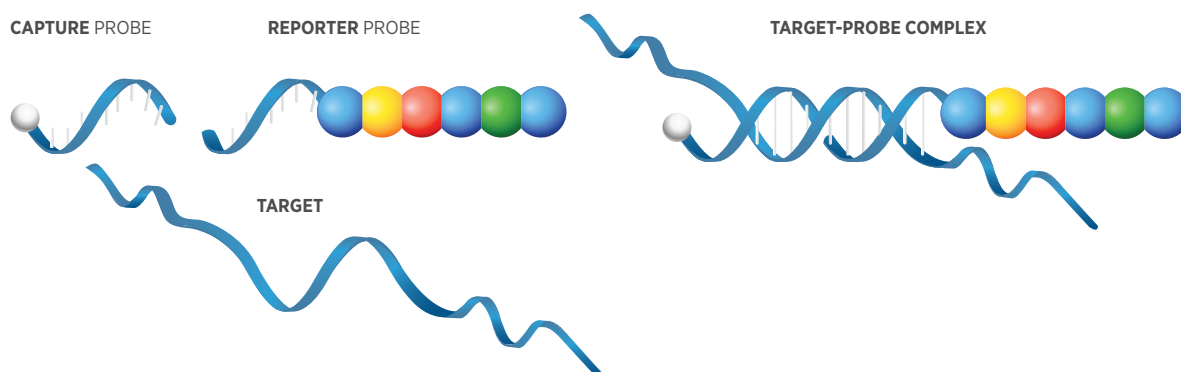
This manual covers the **nCounter Gene Expression Assay** and **Plex² Gene Expression Assay** protocols and provides instruction for both the Total RNA Standard Protocol and the Cell Lysate Protocol. The Total RNA Standard Protocol can also be used with total RNA isolated from blood and Formalin-Fixed, Paraffin-Embedded (FFPE) samples.

NanoString Technology

Principles and Procedures

NanoString's technology is based on digital detection and direct molecular barcoding of target molecules through the use of a color coded probe pair. The probe pair consists of a **Reporter Probe**, which carries the signal on its 5' end, and a **Capture Probe** which carries a biotin on the 3' end. The color codes carry six positions and each position can be one of four colors, thus allowing for a large diversity of tags that can be mixed together in a single well for direct hybridization to target and yet still be individually resolved and identified during data collection.

FIGURE 1.1: Capture and Reporter Probes (left) and, Probe pair bound to an mRNA (right)



Probe pairs are placed into a reaction in massive excess to target RNA or DNA species to ensure that each target finds a probe pair. After hybridization, excess probes are washed away using a two step magnetic bead-based purification on the nCounter® Prep Station.

Magnetic beads derivatized with short nucleic acid sequences that are complementary to the Capture Probe and the Reporter Probes are used sequentially. First, the hybridization mixture is allowed to bind to the magnetic beads by the Capture Probe. Wash steps are performed and excess Reporter Probes and non-target cellular transcripts are removed during wash steps. After washing, the Capture Probes and Target/Probe complexes are eluted off of the beads and are hybridized to magnetic beads complementary to the Reporter Probe. Wash steps are performed and excess Capture Probes are washed away. Finally, the purified Target/Probe complexes are eluted off and are immobilized in the cartridge for data collection.

Data Collection is carried out in the nCounter® Digital Analyzer. At the highest standard data resolution, 555–1155 fields of view (FOV) are collected per flow cell using a microscope objective and a CCD camera yielding data of hundreds of thousands of target molecule counts. Digital images are processed on the nCounter Digital Analyzer and the barcode counts are tabulated in a comma separated value (CSV) format.

The nCounter Analysis System was created by NanoString Technologies. The nCounter system is an easy-to-use integrated system that includes a Prep Station (robot) and a Digital Analyzer (analyzer). The Prep Station and the analyzer together make lab work and sample analysis a simpler process by limiting the variables in experiments for lab technicians. The end result is a very precise and accurate measurement, enabling you to gather data on your targets of interest rapidly with minimal intervention.

nCounter® Expression Assay Overview

The nCounter Expression Assay is run on the nCounter System. The system is comprised of two instruments, the nCounter Prep Station used for post-hybridization processing, and the Digital Analyzer used for data collection. Follow the instructions on the touchscreen to guide you step-by-step through setting up runs on the Prep Station and Digital Analyzer.

FIGURE 1.2: Suggested workflow for the nCounter Expression Assay

Day 1	Manual Processing	Hands-on Time
	Set Up Hybridization	5 minutes
Day 2	Automated Processing	Hands-on Time
	Set Up Prep Station Run	5 minutes
	Set Up Data Collection	5 minutes

Materials Required

The following tables list the recommended materials and instrumentation required to run the nCounter Gene Expression Assay.

TABLE 1.1: Materials required for Total RNA Standard Protocol

Material	Manufacturer	Part Number
nCounter Gene Expression (GX) CodeSet OR nCounter Plex ² Expression Assay Kit	NanoString Technologies	GXA-P1CS-XXX GXA-2PLX-XXX or GXA-4PLX-XXX
nCounter Master Kit (GX only)	NanoString Technologies	NAA-AKIT-XXX
*QIAGEN RNeasy® Kit (or similar)	QIAGEN	74104 or 74106
Disposable gloves	various	
Total RNA - 100ng or 150ng per hybridization assay*		

* If total RNA has been isolated by some other method, please contact NanoString customer support (support@nanosttring.com). We highly recommend verifying the integrity of your total RNA samples via denaturing PAGE or Bioanalyzer (Agilent Technologies) before proceeding with hybridization.

TABLE 1.2: Materials required for Cell Lysate Protocol

Material	Manufacturer	Part Number
nCounter Gene Expression (GX) CodeSet OR nCounter Plex ² Expression Assay Kit	NanoString Technologies	GXA-P1CS-XXX GXA-2PLX-XXX or GXA-4PLX-XXX
nCounter Master Kit	NanoString Technologies	NAA-AKIT-XXX
RLT buffer (or similar)	QIAGEN*	79216
Disposable gloves	various	
Cell lysate 2,500 – 15,000 cells per microliter		

* QIAGEN buffers and a QIAGEN cell lysate procedures (with modifications outlined in Chapter 2) have been tested internally at NanoString Technologies. If using a cell lysis procedure other than QIAGEN'S, please contact NanoString customer support (support@nanosttring.com) for additional information.

TABLE 1.3: Instruments required for Total RNA Standard Protocol and Cell Lysate Protocol

Instrument	Manufacturer	Part Number
NanoDrop ND-1000*	NanoDrop Technologies	n/a
Bioanalyzer 2100*	Agilent	G2940CA
Pipette for 0.5 – 10µL*	Rainin	L-10
Pipette for 2 – 20µL*	Rainin	L-20
Pipette for 20 – 200µL*	Rainin	L-200
Tube-Strip Picofuge*	Stratagene	400540
DNA engine thermocycler, hybridization oven (or similar)	MJ Research / BioRad	PTC-200G†
		PTC-1148†
		PTC-0220G†
		PTC-0221G†
		PTC-0240G†
nCounter Prep Station	NanoString Technologies	NCT-PREP-120
nCounter Digital Analyzer	NanoString Technologies	NCT-DIGA-120
Memory stick	various	n/a

* nCounter system performance data was generated on model PCT DNA engines. Other instruments may produce non-standard results when used with the nCounter assay.

† Any one of these instruments will meet the requirements of the nCounter Assay.

Contact Information

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2 nCounter® Gene Expression Protocols

This chapter outlines the nCounter Gene Expression Assay protocol and provides instruction for both the Total RNA Standard Protocol and the Cell Lysate Protocol. Instruction for setting up 12 nCounter Assays or a single nCounter Assay are provided.

Setting Up 12 nCounter Assays



GENERAL PROBE HANDLING WARNING: During setup of your assay, do not vortex or pipet vigorously to mix as it may shear the Reporter Probes. Mixing should be done by flicking or inverting the tubes. Also, if you use a microfuge to spin down tubes, do not spin any faster than 1,000 rpm for more than 30 seconds and do not “pulse” it to spin because that will cause the centrifuge to go to maximum speed and you may spin your CodeSet out of solution.

The final hybridization reaction will contain the following components: 10µL Reporter CodeSet, 10µL hybridization buffer, a total volume of 5µL of sample RNA (100ng), and 5µL Capture ProbeSet. The order of addition of components is important, please follow the protocol exactly.

1. If following the Total RNA Standard Protocol **go to Step 3.**
2. If following the Cell Lysate Protocol: Lyse Cells according to Qiagen recommendations (see Qiagen RNeasy Mini Handbook, supplied with product numbers 74104 and 74106) with the following modifications:
 - a. Cells should be lysed at concentration between 2,500 and 10,000 cells/µL of RLT buffer. The nCounter cell lysate hybridization procedure has been optimized for ~10,000 mammalian cells/reaction or the equivalent of approximately 100ng of total RNA.
 - b. Cell lysates should be aliquoted and stored at -80°C. Avoid freeze/thaw cycles.
3. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw on ice. Invert several times to mix well and spin down reagent.



IMPORTANT: After it has thawed, inspect the tube of Reporter CodeSet to make sure no colored precipitate is present. If you see a colored precipitate, heat the entire tube to 75°C for 10 minutes and cool at room temperature before using.

4. Create a master mix by adding 130µL of hybridization buffer to the tube of Reporter Probes. Do not remove the Reporter Probes from tube. RNase-free water may also be added to this mix if the volume of the individual RNA samples is less than 5µL and is constant. *(Add enough water for 13 assays to allow one assay's worth of dead volume.)* **Do not add the Capture ProbeSet to the master mix.** Invert to mix and spin down master mix.

5. Label a provided 12 tube strip and cut it in half so it will fit in a picofuge.
6. Add 20µL of mastermix to each of the 12 tubes (*if you added water to the master mix, adjust volumes*). It is advisable to use a fresh tip for each pipetting step to accurately pipet the correct volume. The CodeSet has components that can start to wick up into the tip and not dispense the correct amount if you use the same tip to dispense master mix into all of the hybridization tubes.
7. Add sample according to your protocol type as follows:
 - a. If following the Total RNA Standard Protocol: Add total RNA sample (maximum volume 5µL) for a total of 100ng to each tube.
Go to Step 8.
 - b. If following the Cell Lysate Protocol: Add cell lysate sample (maximum volume 4µL) for a total of approximately 10,000 cells per hybridization assay. Using less than 10,000 cells/reaction will result in fewer counts/gene.
 - c. If using attenuation mix(es), add 1µL of each mix. Note: this reagent can also be added to the master mix if all reactions are to be attenuated.
8. If necessary, add RNase-free water to each tube to bring the volume of each assay to 25µL.
9. Pre-heat thermocycler to 65°C. Program the thermocycler using 30µL volume, calculated temperature, heated lid and “forever” time setting. **Do not set the thermocycler to ramp down to 4°C at the end of the run.**

If a thermocycler is not available, a 65°C hybridization oven may be used. (*The use of a thermocycler is recommended if possible. Due to less stringent temperature control, assay results may be more variable in a hybridization oven.*) To use a hybridization oven, place a large beaker full of water in the oven to ensure a humid environment. **Do not place the samples in the water beaker;** evaporative loss causes the water temperature to be below the air temperature in the oven. Place the samples in a dry rack in the middle of the oven shelf, or tape the strip tubes to a rotator in the center of the oven. Make sure that the strip tubes and/or rack do not touch the sides or bottom of the oven. Failure to follow these instructions may result in uneven hybridization temperatures which can compromise the results.
10. Add 5µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the strip tubes several times and flicking with your finger to ensure complete mixing. Briefly spin down and immediately place the strip tube in the 65°C thermocycler. Minimizing the time between the addition of the Capture ProbeSet and the placement of the reaction at 65°C will increase the sensitivity of your assay.
11. Incubate hybridization assays for at least 12 hours. Hybridizations should be left at 65°C until ready for processing. Maximum hybridization time should not exceed 30 hours.
12. Once removed from the thermocycler, proceed immediately to post-hybridization processing with the nCounter Prep-station. Do not store hybridizations at 4°C.

Setting Up a Single nCounter Assay

During setup of your assay, do not vortex or pipet vigorously to mix as it may shear the Reporter Probes. Mixing should be done by flicking or inverting the tubes. Also, if you use a microfuge to spin down tubes, do not spin any faster than 1,000 rpm for more than 30 seconds and do not “pulse” it to spin because that will cause the centrifuge to go to maximum speed and you may spin your CodeSet out of solution.

The final hybridization reaction will contain the following components: 10µL Reporter CodeSet, 10µL hybridization buffer, a total volume of 5µL of sample RNA (100ng), and 5µL Capture ProbeSet. **If you are making a master mix of any components, DO NOT include the Capture ProbeSet.** It is important that the Capture ProbeSet be added individually to each assay, immediately before the reaction is transferred to 65°C.

1. If following the Total RNA Standard Protocol **go to Step 3.**
2. If following the Cell Lysate Protocol: Lyse Cells according to Qiagen recommendations (see Qiagen RNeasy Mini Handbook, supplied with product numbers 74104 and 74106) with the following modifications:
 - a. Cells should be lysed at concentration between 2,500 and 10,000 cells/µL of RLT buffer. The nCounter cell lysate hybridization procedure has been optimized for ~10,000 mammalian cells/reaction or the equivalent of approximately 100ng of total RNA.
 - b. Cell lysates should be aliquoted and stored at -80°C. Avoid freeze/thaw cycles.
3. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw on ice. Invert several times to mix well and spin down reagent.



IMPORTANT: After it has thawed, inspect the tube of Reporter CodeSet to make sure no colored precipitate is present. If you see a colored precipitate, heat the entire tube to 75°C for 10 minutes and cool at room temperature before using.

4. Label a provided 12 tube strip and cut it in half so it will fit in a microfuge.
5. Add 10µL of Reporter CodeSet reagent to each tube. Store remaining Reporter CodeSet at 4°C for up to 1 month.
6. Add 10µL of hybridization buffer to each tube.
7. Add sample according to your protocol type as follows:
 - a. If following the Total RNA Standard Protocol: Add total RNA sample (maximum volume 5µL) for a total of 100ng to each tube. **Go to Step 8.**
 - b. If following the Cell Lysate Protocol: Add cell lysate sample (maximum volume 4µL) for a total of approximately 10,000 cells per hybridization assay. Using less than 10,000 cells/reaction will result in fewer counts/gene.
 - c. If using attenuation mix(es), add 1µL of each mix.
8. Add RNase-free water to each tube to bring the volume of each assay to 25µL.
9. Pre-heat thermocycler to 65°C. Program the thermocycler using 30µL volume, calculated temperature, heated lid and “forever” time setting. **Do not set the thermocycler to ramp down to 4°C at the end of the run.** Alternatively, a 65°C hybridization oven may be used when a large beaker full of water is placed in the oven to ensure a humid environment.
10. Add 5µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the strip tubes several times and flicking with your finger to ensure complete mixing. Briefly spin down and immediately place the strip tube in the 65°C thermocycler. Minimizing the time between the addition of the Capture ProbeSet and the placement of the reaction at 65°C will increase the sensitivity of your assay.
11. Store remaining Capture ProbeSet at 4°C for up to 1 month.
12. Incubate hybridization assays for at least 12 hours. Hybridizations should be left at 65°C until ready for processing. Maximum hybridization time should not exceed 30 hours.
13. Once removed from the thermocycler, proceed immediately to post-hybridization processing with the nCounter Prep Station. Do not store hybridizations at 4°C.

This section outlines the nCounter Plex² Expression Assay protocol and provides instruction for both the Total RNA Standard Protocol and the Cell Lysate Protocol. Instruction for setting up 24 and 48 Plex² Assays (1 cartridge) is also provided.

Setting Up 24 Plex² Assays (2PLEX)



GENERAL PROBE HANDLING WARNING: During setup of your assay, do not vortex or pipet vigorously to mix as it may shear the Reporter Probes. Mixing should be done by flicking or inverting the tubes. Also, if you use a microfuge to spin down tubes, do not spin any faster than 1,000 rpm for more than 30 seconds and do not “pulse” it to spin because that will cause the centrifuge to go to maximum speed and you may spin your CodeSet out of solution.



IMPORTANT: To facilitate downstream analysis in the nSolver Analysis System, the same sample (Calibration sample) must be run across both CodeSets in the same lane at least once per study (see Figure 2.1). This means that you must hybridize the same Calibration sample in both sub-CodeSets one time. For more information on the Calibration sample see the **nCounter Expression Data Analysis Guidelines**.

FIGURE 2.1: Hybridization setup



To facilitate downstream analysis in the nSolver Analysis System a Calibration sample must be assayed across both Sub-CodeSets in a single lane at least one time per study. In the figure below CS signifies a Sub-CodeSet (CS1-CS2). A single reference sample (red tube marked one) is assayed in each Sub-CodeSet once per study. All other tubes contain unique samples.



IMPORTANT: Be sure to only mix only unique sub-CodeSets in a single tube. Use any given sub-CodeSet only one time in a single lane of the cartridge.

The final hybridization reaction will contain the following components: 10µL Reporter CodeSet, 10µL hybridization buffer, a total volume of 5µL of sample RNA (100ng), and 5µL Capture ProbeSet. The order of addition of components is important, please follow the protocol exactly.

1. If following the Total RNA Standard Protocol **go to Step 3**.
2. If following the Cell Lysate Protocol: Lyse Cells according to Qiagen recommendations (see Qiagen RNeasy Mini Handbook, supplied with product numbers 74104 and 74106) with the following modifications:
 - a. Cells should be lysed at concentration between 2,500 and 10,000 cells/µL of RLT buffer or similar. The nCounter cell lysate hybridization procedure has been optimized for ~10,000 mammalian cells/reaction or the equivalent of approximately 100ng of total RNA.
 - b. Cell lysates should be aliquoted and stored at -80°C. Avoid freeze/thaw cycles.
3. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw on ice. Invert several times to mix well and spin down reagent.



IMPORTANT: After it has thawed, inspect the tube of Reporter CodeSet to make sure no colored precipitate is present. If you see a colored precipitate, heat the entire tube to 75°C for 10 minutes and cool at room temperature before using.

4. Create a master mix by adding 130µL of hybridization buffer to the tube of Reporter Probes. Do not remove the Reporter Probes from tube. RNase-free water may also be added to this mix if the volume of the individual RNA samples is less than 5µL and is constant. (*Add enough water for 13 assays to allow one assay's worth of dead volume.*) **Do not add the Capture ProbeSet to the master mix.** Invert to mix and spin down master mix.
5. Label two provided 12 tube strip tubes and cut each in half so it will fit in a picofuge.
6. Add 20µL of mastermix to each of the 24 tubes (*if you added water to the master mix, adjust volumes*). It is advisable to use a fresh tip for each pipetting step to accurately pipet the correct volume. The CodeSet has components that can start to wick up into the tip and not dispense the correct amount if you use the same tip to dispense master mix into all of the hybridization tubes.
7. Add sample according to your protocol type as follows:
 - a. If following the Total RNA Standard Protocol: Add total RNA sample (maximum volume 5µL) for a total of 100ng to each tube. Go to Step 8.
 - b. If following the Cell Lysate Protocol: Add cell lysate sample (maximum volume 4µL) for a total of approximately 10,000 cells per hybridization assay. Using less than 10,000 cells/reaction will result in fewer counts/gene.
 - c. If using attenuation mix(es), add 1µL of each mix. Note: this reagent can also be added to the master mix if all reactions are to be attenuated.
8. If necessary, add RNase-free water to each tube to bring the volume of each assay to 25µL.
9. Pre-heat thermocycler to 65°C. Program the thermocycler using 30µL volume, calculated temperature, heated lid and “forever” time setting. **Do not set the thermocycler to ramp down to 4°C at the end of the run.**

If a thermocycler is not available, a 65°C hybridization oven may be used. (*The use of a thermocycler is recommended if possible. Due to less stringent temperature control, assay results may be more variable in a hybridization oven.*) To use a hybridization oven, place a large beaker full of water in the oven to ensure a humid environment. **Do not place the samples in the water beaker;** evaporative loss causes the water temperature to be below the air temperature in the oven. Place the samples in a dry rack in the middle of the oven shelf, or tape the strip tubes to a rotator in the center of the oven. Make sure that the strip tubes and/or rack do not touch the sides or bottom of the oven. Failure to follow these instructions may result in uneven hybridization temperatures which can compromise the results.
10. Add 5µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the strip tubes several times and flicking with your finger to ensure complete mixing. Briefly spin down and immediately place the strip tube in the 65°C thermocycler. Minimizing the time between the addition of the Capture ProbeSet and the placement of the reaction at 65°C will increase the sensitivity of your assay.
11. Incubate hybridization assays for at least 12 hours. Hybridizations should be left at 65°C until ready for processing. Maximum hybridization time should not exceed 30 hours.
12. Remove tubes from the thermocycler, and combine full hybridization volumes of strip tubes 1 and 2 into strip tube 1, as shown in **Figure 2.2**, maintaining tube orientation. The resulting final volume is 60µL per well.
13. Once removed from the thermocycler, recap tubes, briefly spin, and proceed immediately to post-hybridization processing with the nCounter Prep Station. Do not store hybridizations at 4°C.

FIGURE 2.2: Post-hybridization mixing step



Post-hybridization, add full hybridization reaction volume from CS2 into the CS1 tube, maintaining tube orientation. The resulting final volume is 60µL per well. In the figure above, hybridization volumes from CS2 are combined into the CS1 strip tube. The CS1 strip tube is then recapped and briefly spun before loading into the Prep Station.

Setting Up 48 Plex² Assays (4PLEX)

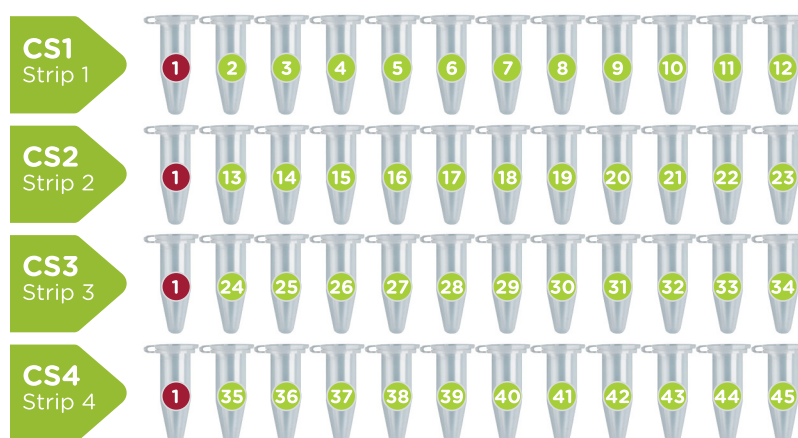


GENERAL PROBE HANDLING WARNING: During setup of your assay, do not vortex or pipet vigorously to mix as it may shear the Reporter Probes. Mixing should be done by flicking or inverting the tubes. Also, if you use a microfuge to spin down tubes, do not spin any faster than 1,000 rpm for more than 30 seconds and do not “pulse” it to spin because that will cause the centrifuge to go to maximum speed and you may spin your CodeSet out of solution.



IMPORTANT: To facilitate downstream analysis in the nSolver Analysis System, the same sample (Calibration sample) must be run across both CodeSets in the same lane at least once per study (see Figure 2.3). This means that you must hybridize the same Calibration sample in both sub-CodeSets one time. For more information on the Calibration sample see the **nCounter Expression Data Analysis Guidelines**.

FIGURE 2.3: Hybridization setup



To facilitate downstream analysis in the nSolver Analysis System a Calibration sample must be assayed across all four Sub-CodeSets in a single lane at least one time per study. In the figure below CS signifies a Sub-CodeSet (CS1-CS4). A single Calibration sample (red tube marked one) is assayed in each Sub-CodeSet at least once per study. All other tubes contain unique samples.



IMPORTANT: Be sure to only mix only unique sub-CodeSets in a single tube. Use any given sub-CodeSet only one time in a single lane of the cartridge.

The final hybridization reaction will contain the following components: 10µL Reporter CodeSet, 10µL hybridization buffer, a total volume of 5µL of sample RNA (150ng), and 5µL Capture ProbeSet. The order of addition of components is important, please follow the protocol exactly.

1. If following the Total RNA Standard Protocol go to **Step 3**.
2. If following the Cell Lysate Protocol: Lyse Cells according to Qiagen recommendations (see Qiagen RNeasy Mini Handbook, supplied with product numbers 74104 and 74106) with the following modifications:
 - a. Cells should be lysed at concentration between 3,500 and 15,000 cells/µL of RLT buffer. The nCounter Plex² cell lysate hybridization procedure has been optimized for ~15,000 mammalian cells/reaction or the equivalent of approximately 150ng of total RNA.
 - b. Cell lysates should be aliquoted and stored at -80°C. Avoid freeze/thaw cycles.

3. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw on ice. Invert several times to mix well and spin down reagent.

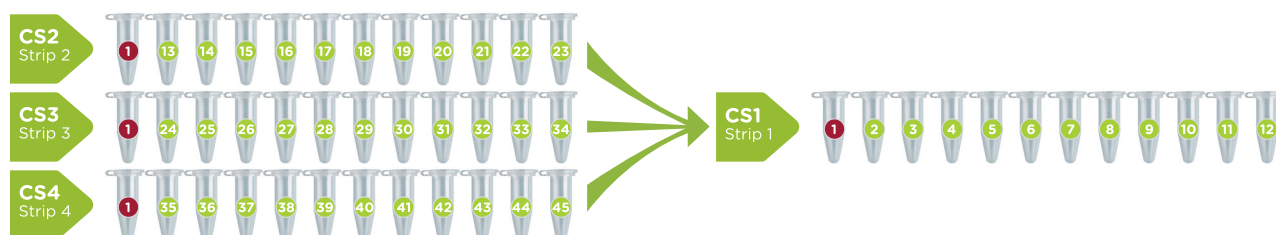


IMPORTANT: After it has thawed, inspect the tube of Reporter CodeSet to make sure no colored precipitate is present. If you see a colored precipitate, heat the entire tube to 75°C for 10 minutes and cool at room temperature before using.

4. Create a master mix for each sub-CodeSet by adding 130µL of hybridization buffer to the tube of Reporter Probes for each CodeSet. Do not remove the Reporter Probes from tube. RNAse-free water may also be added to this mix if the volume of the individual RNA samples is less than 5µL and is constant. *(Add enough water for 13 assays to allow one assay's worth of dead volume.)*
Do not add the Capture ProbeSet to the master mix. Invert to mix and spin down master mix.
5. Label four provided 12 tube strip tubes and cut each in half so it will fit in a picofuge.
6. Add 20µL of mastermix to each of the tubes *(if you added water to the master mix, adjust volumes)*. It is advisable to use a fresh tip for each pipetting step to accurately pipet the correct volume. The CodeSet has components that can start to wick up into the tip and not dispense the correct amount if you use the same tip to dispense master mix into all of the hybridization tubes.
7. Add sample according to your protocol type as follows:
 - a. If following the Total RNA Standard Protocol: Add total RNA sample (maximum volume 5µL) for a total of 150ng to each tube. Go to Step 8.
 - b. If following the Cell Lysate Protocol: Add cell lysate sample (maximum volume 4µL) for a total of approximately 15,000 cells per hybridization assay. Using less than 15,000 cells/reaction will result in fewer counts/gene.
 - c. If using attenuation mix(es), add 1µL of each mix. Note: this reagent can also be added to the master mix if all reactions are to be attenuated.
8. If necessary, add RNAse-free water to each tube to bring the volume of each assay to 25µL.
9. Pre-heat thermocycler to 65°C. Program the thermocycler using 30µL volume, calculated temperature, heated lid and "forever" time setting. **Do not set the thermocycler to ramp down to 4°C at the end of the run.**
If a thermocycler is not available, a 65°C hybridization oven may be used. *(The use of a thermocycler is recommended if possible. Due to less stringent temperature control, assay results may be more variable in a hybridization oven.)* To use a hybridization oven, place a large beaker full of water in the oven to ensure a humid environment. **Do not place the samples in the water beaker;** evaporative loss causes the water temperature to be below the air temperature in the oven. Place the samples in a dry rack in the middle of the oven shelf, or tape the strip tubes to a rotator in the center of the oven. Make sure that the strip tubes and/or rack do not touch the sides or bottom of the oven. Failure to follow these instructions may result in uneven hybridization temperatures which can compromise the results.
10. Add 5µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the strip tubes several times and flicking with your finger to ensure complete mixing. Briefly spin down and immediately place the strip tube in the 65°C thermocycler. Minimizing the time between the addition of the Capture ProbeSet and the placement of the reaction at 65°C will increase the sensitivity of your assay.
11. Incubate hybridization assays for at least 12 hours. Hybridizations should be left at 65°C until ready for processing. Maximum hybridization time should not exceed 30 hours.

12. Remove tubes from the thermocycler, and combine full hybridization volumes of strip tubes CS2, CS3, and CS4 into CS1 (strip tube 1), as shown in **Figure 2.4**, maintaining tube orientation. The resulting final volume is 120µL per well.
13. Once removed from the thermocycler, recap, and briefly spin tubes before proceeding immediately to post-hybridization processing with the nCounter Prep Station. Do not store hybridizations at 4°C.

FIGURE 2.4: Post-hybridization mixing step



Post-hybridization, add full hybridization reaction volume from CS2, CS3, and CS4 into the CS1 tube, maintaining tube orientation. The resulting final volume is 120µL per well. In the figure above, hybridization volumes from CS1, CS2, and CS3 are combined into the CS1 strip tube. The CS1 strip tube is then recapped, briefly spun, and loaded into the Prep Station.



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